Thiols as markers of redox status in type 1 diabetes mellitus

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Abstract

Introduction: Type 1 diabetes mellitus (T1DM) is associated with inflammation and the production of reactive oxygen species (ROS). Systemically, free thiols (R-SH) can be oxidized by ROS and circulating R-SH concentrations may directly reflect the systemic redox status. In this study the association between R-SH and clinical parameters of T1DM, including glycated haemoglobin A1c (HbA1c), was investigated. This is of particular interest since thiols are amendable to therapeutic intervention.

Methods: As part of a prospective cohort study, data from 216 patients with a mean age of 45 (12) years, 57% male, diabetes duration 22 (16, 30) years and HbA1c of 60 (11) mmol/mol were examined. Baseline data were collected in 2002 and follow-up data in 2018. Cox proportional hazards regression analysis, with age, sex, HbA1c and R-SH, was used to assess prognostic factors for the development of complications.

Results: At baseline, the plasma concentration of R-SH was 281.8 ± 34.0 µM. In addition to a lower concentration of NT-proBNP in the highest R-SH quartile (305–379 µM) there were no differences in baseline characteristics between the quartiles of R-SH. The Pearson correlation coefficient for R-SH and NT-proBNP was –0.290 ($p < 0.001$). No significant correlation between R-SH and baseline HbA1c ($r = -0.024$, $p = 0.726$) was present. During follow-up, 42 macrovascular and 92 microvascular complications occurred. In Cox regression, R-SH was not a prognostic factor for the development of microvascular [hazard ratio (HR) 0.999 (95% confidence interval (CI) 0.993, 1.005)] and macrovascular [HR 0.993 (95% CI 0.984, 1.002)] complications.

Conclusions: In addition to a negative association with NT-proBNP, no relevant relationships between R-SH and parameters of T1DM, including HbA1c, were present in this study.

Keywords: free sulfhydryl, glycaemia, oxidative stress, thiols, type 1 diabetes

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Introduction

Systemic oxidative stress is caused by an imbalance between oxidative and antioxidative systems, resulting in a net overload of oxidative free radicals and thereby reactive oxygen species (ROS).[1] As a pathogenic result, these excessive amounts of ROS modify the function and structure of cellular components such as proteins and lipids. This leads to cellular dysfunction, as evidenced by an impairment of energy metabolism, changes in cell signalling, variations in cell cycle control and an impairment of cell transport mechanisms.[2] Subsequently, a dysfunction in processes such as immune activation and inflammation occur. Hyperglycaemia by itself is able to promote the production of ROS as revealed by an enhancement in lipid peroxidation.[2,3] These processes are considered central in the pathogenesis of type 1 diabetes mellitus (T1DM) related microvascular and macrovascular complications.[4] Despite improvements in the management of glycaemia and cardiovascular risk factors,

T1DM is still accompanied by a high rate of complications, all of which are associated with mortality as well as reduced health-related quality of life (HRQOL).[5]

Thiols are organic compounds that are characterized by the presence of a sulfhydryl (R-SH) moiety.[6] Thiols exist as proteins with one (or more) free cysteine groups or as low-molecular-weight compounds like glutathione in cells and also in extracellular fluids. Systemically, the concentration of thiols is lower than intracellularly. In serum, albumin is the most abundant thiol.[7] R-SH groups can be oxidized by ROS and by other reactive species such as sulphur and nitrogen species. The plasma concentrations of total R-SH are proposed to reflect the systemic body redox status indicating that a decline in circulating R-SH reveals enhancement of the oxidative tone.[8,9] Free thiols are receptive to therapeutic modulation, for example by cysteine derivatives such as N-acetylcysteine. Evaluation of systemic free thiols in disease conditions may therefore have diagnostic or therapeutic merit.[7,10,11]

Serum free thiol depletion has been reported in patients with cardiovascular disease, including acute myocardial infarction (MI), when compared with controls.[12,13] In stable heart failure patients, higher levels of serum free thiols were associated with favourable diseases outcomes.[9] In addition, high R-SH serum concentrations have been shown to be associated with a beneficial cardiovascular risk profile and a better patient and graft survival in renal transplant recipients.[14] Interestingly, in inflammatory bowel disease (IBD) free thiols are a better reflection of the disease activity as determined by endoscopy, then faecal calprotectin, the classical biomarker for IBD.[15] Recently, increased oxidation of thiols towards the disulphide form was recently found in T1DM patients as compared with healthy subjects.[16] In that analysis, the total thiol concentration was correlated negatively with C-reactive protein and glycated haemoglobin A1c (HbA1c).

Given these results, and the potential of R-SH as a modifiable marker of ROS-mediated damage in the progression of T1DM and associated complications, we hypothesized that high R-SH concentrations are positively associated with clinical parameters of T1DM. Therefore, we studied R-SH in a cohort of T1DM patients.

Patients and methods

The FANTA study was designed as a prospective, cohort study to investigate several disease factors, including oxidative stress and HRQOL in persons with T1DM. Full study design and the results of HRQOL analysis in a subset of patients have been published in detail previously.[17,18] In brief, from January 1995 to January 1996, consecutive visiting T1DM patients treated at the diabetes outpatient clinic of the Weezenlanden Hospital (nowadays Isala; Zwolle, The Netherlands) were invited to participate in the study. T1DM was defined as starting insulin therapy within 6 months after the first signs of diabetes and before the age of 30 years, or the absence of C-peptide secretion. In total, 293 patients agreed to participate. In the period from 1996 to 2002, a total of 32 patients dropped out of the study. Reasons for dropping out were: moving out of the area or referral to another physician ($n = 12$), unknown ($n = 10$), lack of interest ($n = 6$), death ($n = 2$) and incorrect diagnosis of T1DM ($n = 2$). For the present analyses, we analysed the 261 patients who were participating in 2002. For 45 patients, no serum samples were available. Therefore, a total of 216 patients were included in the present analysis.

The primary objective of the present study was to investigate the transversal association of serum R-SH concentrations and HbA1c in T1DM. As a secondary outcome, association of R-SH with other clinical characteristics of T1DM was assessed. Furthermore, the longitudinal association of serum R-SH concentrations with the development of microvascular and macrovascular complications was investigated.

At baseline, aliquots of ethylenediaminetetraacetic acid (EDTA) samples were collected and stored at –80°C (without thawing) until measurement. R-SH were measured as described previously, with minor modifications.[19,20] Briefly, 75 µl serum was diluted 1:4 in 0.1 M Tris buffer (pH 8.2) and transferred to a 96-well plate. Using a Sunrise microplate reader (Tecan AG, Männedorf, Switzerland), background absorption was measured at 412 nm with a reference filter at 630 nm. Subsequently, 10 µl 3.8 mM 5,5′-dithiobis(2-nitrobenzoic acid) (Sigma-Aldrich, Zwijndrecht, Netherlands) in 0.1 M phosphate buffer (pH 7) was added to the samples. Following 20 min of incubation at room temperature, absorption was

read again. The concentration of R-SH in the samples was determined by comparing their absorbance readings with a standard curve of L-cysteine (15–1000 μM; Fluka Biochemika, Buchs, Switzerland) in 0.1 M Tris and 10 mM EDTA (pH 8.2).

Data concerning demographics, mode of therapy, height, weight, presence of complications, blood pressure and laboratory measurements were collected annually during follow-up according to a standardized protocol and standardized forms. For the analysis with respect to complications and vital status follow-up data from the year 2018 was gathered. In addition to the standardized forms an additional search concerning complications and vital status was performed using electronic hospital records. HbA1c is expressed in SI, International Federation of Clinical Chemistry and Laboratory Medicine recommended (as mmol/mol) units.

Macrovascular complications were defined as angina pectoris (AP), peripheral artery disease (PAD), MI, percutaneous transluminal coronary angioplasty (PTCA), coronary artery bypass grafting (CABG), cerebrovascular accident (CVA) or transient ischemic attack (TIA). Microvascular complications were defined as diabetic retinopathy, albuminuria (both microalbuminuria and macroalbuminuria) and diabetic peripheral neuropathy. Microalbuminuria was defined as 20–200 mg/l albumin or an albumin–creatinine ratio between 2.5 and 25 mg/mmol (22–221 mg/g) in men and 3.5 and 35 mg/mmol (31–310 mg/g) in women. Macroalbuminuria was defined as >200 mg/l albumin or a albumin–creatinine ratio greater than 25 mg/mmol and 35 mg/mmol for men and women, respectively.[21] An ophthalmologist determined the presence of diabetic retinopathy biannually. Foot sensibility was tested with 5.07 Semmes–Weinstein monofilaments. Neuropathy was defined as two or more errors in a test of three, affecting at least one foot.

Descriptive summaries included the mean with standard deviation (SD) for normally distributed variables and the median with the interquartile range (25th–75th percentile) for other variables. Baseline data were compared with the chi-squared test in case of categorical data. In case of continuous data, Student's *t* test or Mann–Whitney *U* test were used if the data was distributed normally or skewed, respectively. *Q–Q* plots and histograms were used to determine whether the tested variable had a normal distribution or not. The Pearson correlation coefficient was used to investigate the relation between baseline R-SH and HbA1c concentrations. To visualize the relation of the tertiles of R-SH with the development of complications and death during follow-up, a Kaplan–Meier curve was constructed. Differences in time until occurrence of any complications were assessed for statistical significance using the log-rank test. Cox proportional hazards regression analysis, with age, sex, HbA1c and R-SH, was used to assess prognostic factors for the development of microvascular or macrovascular complication. A (two-sided) p value of less than 0.05 was considered statistically significant.

All analyses were performed using SPSS version 24.0 (SPSS Inc. Chicago, IL, USA). A (two-sided) p value of less than 0.05 was considered statistically significant. The study was performed in accordance with the Declaration of Helsinki. Written and oral informed consent was obtained from all patients, and the local medical ethics committee of Isala (METC Isala, Zwolle, the Netherlands) approved the protocol.

Results

Baseline characteristics of the 216 patients with T1DM are presented in Table 1. The cohort had a mean age of 45.1 (11.6) years, 57% were male, diabetes duration was 22.1 (15.8, 30.4) years and HbA1c concentration was 60 (11) mmol/mol. At baseline, there were 110 (51%) patients with a microvascular complication and 22 (10%) patients with macrovascular complications. Among all patients, R-SH concentrations were normally distributed, with a mean concentration of 281.8 (34.0) μM.

Concentrations of R-SH did not differ between men and woman [281.5 μM (255.0, 301.8), 287.5 μM (260.0, 309.8), $p = 0.44$]. In addition to a lower concentration of NT-proBNP in the highest quartile of R-SH (305–379 μM) there were no differences in baseline characteristics between the quartiles of R-SH (Table 1). The Pearson correlation coefficient for R-SH and NT-proBNP was –0.290 ($p < 0.001$). Of note, there was no significant correlation between R-SH and HbA1c at baseline ($r = -0.024$, $p = 0.726$).

During the follow-up duration of 15.4 (7.4, 15.8) years, one patient died (noncardiovascular cause)

Table 1. Baseline characteristics of all patients and per quartile of R-SH.

	All	Quartiles of R-SH			
	n = 216	172–257 µM *n* = 54	258–284 µM *n* = 51	285–304 µM *n* = 58	305–379 µM *n* = 53
Demographics					
Age (years)	45.1 (11.6)	46.3 (7.3)	44.6 (9.5)	44.4 (12.5)	45.2 (15.4)
Diabetes duration (years)	22.1 (15.8, 30.4)	23.4 (15.2, 31.3)	21.4 (16.4, 32.9)	20.7 (14.6, 30.1)	24.3 (16.5, 29.5)
Male gender (*n*)	123 (57.4)	33 (61.1)	32 (62.7)	34 (58.6)	25 (47.2)
BMI (kg/m^2)	25.4 (23.3, 28.4)	25.5 (23.1, 28.6)	25.2 (22.8, 28.6)	25.7 (23.3, 29.0)	25.5 (23.6, 28.3)
Systolic blood pressure (mmHg)	130.4 (17.6)	132.6 (19.2)	129.1 (15.2)	131.4 (17.2)	128.3 (18.6)
Diastolic blood pressure (mmHg)	77.3 (10.2)	77.9 (10.1)	76.1 (10.4)	80.0 (10.3)	74.8 (9.3)
Mode of insulin administration: MDI	124 (57.4)	35 (64.8)	24 (47.1)	38 (65.5)	27 (50.9)
Mode of insulin administration: CSII	92 (42.6)	19 (35.2)	27 (52.9)	20 (34.5)	26 (49.1)
Total insulin dose§	58.8 (19.6)	60.3 (20.2)	49.5 (16.0)	60.9 (19.5)	39.3 (17.9)
Complications					
Microvascular complications					
Retinopathy (*n*)	92 (42.6)	26 (48.1)	22 (43.1)	20 (34.5)	24 (45.3)
Neuropathy (*n*)	24 (11.1)	3 (5.6)	6 (11.8)	7 (12.1)	8 (15.1)
Micro albuminuria (*n*)	28 (13.0)	10 (18.5)	5 (9.8)	8 (13.8)	5 (9.4)
Macro albuminuria (*n*)	8 (3.7)	5 (9.3)	0 (0.0)	1 (1.7)	2 (3.8)
Macrovascular events					
Angina pectoris (*n*)	3 (1.4)	2 (3.7)	0 (0.0)	1 (1.7)	0 (0.0)
MI (*n*)	3 (1.4)	0 (0.0)	0 (0.0)	1 (1.7)	2 (3.8)
PTCA (*n*)	3 (1.4)	1 (1.9)	0 (0.0)	2 (3.4)	0 (0.0)
PAD (*n*)	5 (2.3)	1 (1.9)	2 (3.9)	0 (0.0)	2 (3.8)
CABG (*n*)	3 (1.4)	0 (0.0)	1 (2.0)	2 (3.4)	0 (0.0)
TIA (*n*)	2 (0.9)	0 (0.0)	1 (2.0)	1 (1.7)	(0.0)
CVA (*n*)	4 (1.9)	1 (1.9)	1 (2.0)	1 (1.7)	1 (1.9)
Laboratory measurements					
R-SH (µM)	281.8 (34.0)	237.0 (17.1)	271.4 (7.0)	294.4 (6.3)	323 (15.4)
HbA1c (%)	7.6 (1.0)	7.7 (1.1)	7.5 (1.1)	7.6 (1.1)	7.6 (1.0)
Creatinine (µmol/l)	85.0 (77.0, 94.0)	84.0 (76.0, 93.5)	86.5 (77.8, 93.0)	84.0 (78.0, 94.0)	85.0 (78.0, 95.0)

(Continued)

Table 1. (Continued)

	All	Quartiles of R-SH			
	n **= 216**	**172–257 µM *n*** **= 54**	**258–284 µM *n*** **= 51**	**285–304 µM *n*** **= 58**	**305–379 µM *n*** **= 53**
eGFR (MDRD, ml/min/1.73 m^2)	126.1 (109.4, 141.7)	105.5 (91.7, 113.4)	105.9 (77.9, 127.9)	111.3 (86.8, 122.4)	103.3 (83.5, 131.0)
Total cholesterol (mmol/l)	4.6 (1.0)	4.6 (1.0)	4.7 (0.9)	4.4 (0.9)	4.8 (1.0)
HDL cholesterol (mmol/l)	1.5 (0.4)	1.6 (0.4)	1.4 (0.4)	1.5 (0.4)	1.6 (0.5)
Total cholesterol:HDL ratio	3.0 (1.0, 3.0)	3.1 (2.3, 3.9)	3.2 (2.6, 4.0)	2.8 (2.3, 3.4)	3.1 (2.6, 4.1)
LDL cholesterol (mmol/l)	2.6 (0.9)	2.5 (1.0)	2.7 (0.7)	2.4 (0.8)	2.7 (0.9)
Triglycerides (mmol/l)	0.9 (0.7, 1.4)	1.0 (0.7, 1.4)	1.0 (0.7, 1.4)	0.8 (0.7, 1.2)	1.0 (0.7, 1.7)
C-reactive protein (mg/l)	2.0 (1.0, 3.0)	2.0 (1.0, 4.3)	1.0 (1.0, 3.0)	2.0 (1.0, 4.0)	2.0 [1.0, 3.0]
NT-proBNP (pmol/l)	40.5 (16.2, 86.2)	73.0 (40.9, 41.7)	45.2 (17.0, 85.7)	29.0 (14.2, 66.9)	25.6 (8.7, 49.4)*

In the second-row, data are presented as number (%), mean (SD) or median (IQR).
*$p < 0.05$, §$n = 29$.
BMI, body mass index; CABG, coronary artery bypass grafting; CSII, continuous subcutaneous insulin infusion; CVA, cerebral vascular event; eGFR, estimated glomerular filtration rate; HbA1c, glycated haemoglobin A1c; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MDI, multiple daily injections; MDRD, modification of diet in renal disease; MI, myocardial infarction; NA, not applicable; PAD, peripheral artery disease; PTCA, percutaneous transluminal coronary angioplasty; R-SH, total free thiol groups; TIA, transient ischemic attack.

and 42 patients experienced a total of 76 new macrovascular complications: 17 MI, 17 CVA, 17 PAD, 11 PTCA, 7 CABG, 4 AP and 3 TIA. A total of 92 (43%) patients developed a microvascular complication: neuropathy ($n = 36$), retinopathy ($n = 55$), microalbuminuria ($n = 19$) or macroalbuminuria ($n = 6$). There was no difference in baseline R-SH concentration at baseline between patients with or without the development of a microvascular or macrovascular complication during follow-up: 287.0 (258.0, 306.0) µM for persons without *versus* 281.0 (254.3, 303.3) µM for persons with a microvascular complication. For macrovascular complications these numbers were 270.0 (249.8, 300.2) µM for persons without *versus* 286.0 (261.5, 306.0) µM for persons with macrovascular complications.

Overall, the time until the first event was 11.1 [95% confidence interval (CI) 9.7–12.5] years. As presented in Figure 1 there were no differences between the quartiles of R-SH in the development of (both microvascular and macrovascular) complications during follow-up ($p = 0.62$).

Cox regression analysis demonstrated that R-SH was not a prognostic factor for the development of both microvascular and macrovascular complications (see Table 2).

Discussion

There were no relevant relationships at baseline between circulating R-SH concentrations and indices of T1DM management, including HbA1c, and cardiovascular risk factors in this cohort of 216 patients with T1DM. Although a negative correlation of R-SH with NT-proBNP concentrations was present at baseline, we did not observe this for HbA1c.

These findings are opposite to the significant relation between fasting blood glucose, HbA1c, C-reactive protein with disulphide/native thiol and disulphide/total thiol levels found by Ates and colleagues.[16] In their study a shift of dynamic thiol/disulphide homeostasis toward the disulphide form was present in T1DM ($n = 38$) patients as compared with healthy controls ($n = 38$). This shift may be secondary to increased ROS formation owing to (hyperglycaemia induced) inflammation in patients with T1DM. It could be hypothesized that the discrepancy between these and our findings is related to the degree of dysglycaemia; in the

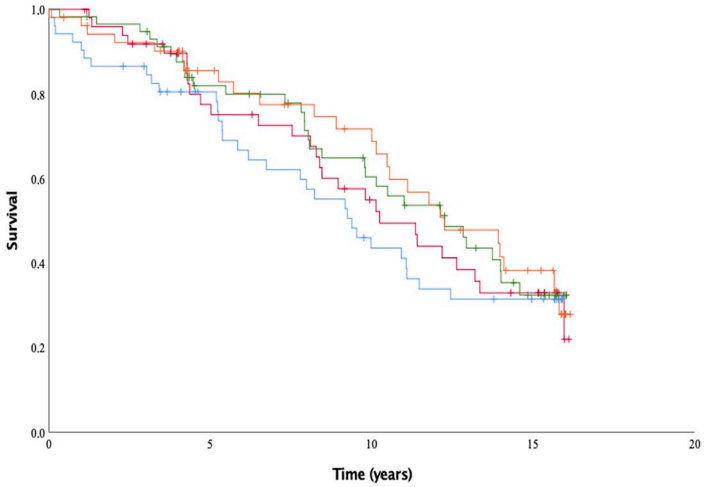

Figure 1. Time between start of the study and occurrence of first complication for different quartiles of R-SH. The blue line represents the lowest [172–257 µM; median time to event 9.4 (95% confidence interval (CI) 7.2–11.6) years], the red [258–284 µM; 10.2 (95% CI 7.5–13.0) years) and green (285–304 µM; 12.3 (95% CI 9.4–15.1) years] lines the middle quartile and the orange [305–379 µM; 12.3 (95% CI 8.5–16.1) years] the highest quartile of free thiols (R-SH).

Table 2. Multivariable Cox regression analysis.

	HR (95% CI) for microvascular complications	HR (95% CI) for macrovascular complications
Age	1.025 (1.005, 1.046)	1.071 (1.040, 1.103)
Gender (male)	1.178 (0.762, 1.822)	2.945 (1.292, 6.710)
HbA1c	1.357 (1.100, 1.674)	1.066 (0.787, 1.444)
R-SH	0.999 (0.993, 1.005)	0.993 (0.984, 1.002)

Data are presented as hazard ratio (HR) [95% confidence interval (CI)]. Number of microvascular events: 92. Number of macrovascular events: 42.
HbA1c, glycated haemoglobin A1c; R-SH, total free thiol groups.

study by Ates and colleagues mean HbA1c was 88 mmol/mol as compared with 60 mmol/mol in the present study. Another explanation is that thiol metabolism is mostly influenced by short-term oxidative stress caused by acute glycaemic derangements. This may indicate that R-SH merely provide a snapshot of someone's momentary ROS/antioxidant balance, which is then unlikely to correlate with a long-term indicator of glycaemic control such as HbA1c. Therefore, it would be of interest to investigate the within-person relationship between R-SH and within-day glycaemic variability (using continuous glucose measurements) and insulin administration as both instantaneously influences oxidative stress. Unfortunately, the present study lacks this information.

In the present study a negative correlation between R-SH and NT-proBNP, a marker of volume expansion and heart failure, was found. This observation is in agreement with previous studies among persons with heart failure and a renal transplant.[9,14] Of note, this correlation remained present after adjustment for potential confounders (see Table 3). Increased ROS are an important element of the pathophysiology of heart failure, and contributes to the development of myocardial and vascular dysfunction; therefore, it is conceivable that thiols are a reflection of these processes.[22] On the other hand, effects on volume expansion cannot be ruled out. Since T1DM is accompanied by an increased prevalence of heart failure,[23] the nature of the relation

Table 3. Multivariate linear regression analysis for R-SH.

	St. beta	95% CI	*p*
Gender (1 = male)	0.114	−2.2, 17.5	0.129
Creatinine (μmol/l)	0.051	−0.2, 0.5	0.489
Log BMI (kg/m^2)	0.017	−27.9, 35.2	0.807
Log NTproBNP	−0.410	−15.2, −7.6	<0.001
Presence of macrovascular complication (1 = yes)	0.038	−12.2, 21.6	0.588

BMI, body mass index; CI, confidence interval; R-SH, total free thiol groups; St. beta, Standardized beta (regression) coefficient

between R-SH and NT-proBNP needs to be further explored.

For the interpretation of the results of this study, several limitations should be taken into account including the limited sample size. Although this study currently has the largest population with the longest follow-up concerning R-SH in T1DM in the literature, we cannot rule out that the longitudinal analyses were underpowered. In addition, as only one baseline measurement of R-SH was used for these analyses and there was a lack of data regarding other parameters over time (including HbA1c), these analyses should be interpreted with caution. Furthermore, the lack of a nondiabetic reference population, amount of drop-out of the original cohort and the lack of other plasma antioxidant species such as ascorbate, uric acid and small-molecular-weight thiols and markers of inflammation should be mentioned.

In conclusion, no relevant relationships between R-SH and parameters of T1DM care were present in this study. Although the results of this small study needs confirmation, it could be hypothesized that R-SH is more appropriate as an instantaneous, rather than a long-term, read-out of oxidative stress in T1DM.

Authors' note

Conceptualization: PvD, FW, HvG and HB. Methodology: PvD, SO, AP, AEA,FW, HvG and HB; Software, PvD. Validation: PvD, AP and HvG. Formal Analysis: PvD. Investigation: PvD and FW. Resources: HB, FW, PvD and HvG; Data Curation: PvD; Writing – Original Draft Preparation: PvD. Writing – Review & Editing: PvD, FW, SO, AEA, AP, MM, HB, HvG. Visualization: PvD. Supervision: PvD, HvG and HB. Project Administration: PvD. Funding Acquisition: HB, FW, PvD and HvG.

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Conflict of interest statement

The authors declare that they have no conflict of interest.

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